

A dominant-negative mutant of human poly(ADP-ribose) polymerase affects cell recovery, apoptosis, and sister chromatid exchange following DNA damage

(cellular response to DNA damage/DNA repair/G₂ arrest)

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ABSTRACT Poly(ADP-ribose) polymerase [PARP; NAD⁺ ADP-ribosyltransferase; NAD⁺:poly(adenosine-diphosphate-D-ribosyl)-acceptor ADP-D-ribosyltransferase, EC 2.4.2.30] is a zinc-dependent eukaryotic DNA-binding protein that specifically recognizes DNA strand breaks produced by various genotoxic agents. To study the biological function of this enzyme, we have established stable HeLa cell lines that constitutively produce the 46-kDa DNA-binding domain of human PARP (PARP-DBD), leading to the trans-dominant inhibition of resident PARP activity. As a control, a cell line was constructed, producing a point-mutated version of the DBD, which has no affinity for DNA *in vitro*. Expression of the PARP-DBD had only a slight effect on undamaged cells but had drastic consequences for cells treated with genotoxic agents. Exposure of cell lines expressing the wild-type (wt) or the mutated PARP-DBD, with low doses of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) resulted in an increase in their doubling time, a G₂ + M accumulation, and a marked reduction in cell survival. However, UVC irradiation had no preferential effect on the cell growth or viability of cell lines expressing the PARP-DBD. These PARP-DBD-expressing cells treated with MNNG presented the characteristic nucleosomal DNA ladder, one of the hallmarks of cell death by apoptosis. Moreover, these cells exhibited chromosomal instability as demonstrated by higher frequencies of both spontaneous and MNNG-induced sister chromatid exchanges. Surprisingly, the line producing the mutated DBD had the same behavior as those producing the wt DBD, indicating that the mechanism of action of the dominant-negative mutant involves more than its DNA-binding function. Altogether, these results strongly suggest that PARP is an element of the G₂ checkpoint in mammalian cells.

DNA is continually damaged by environmental genotoxic agents and by endogenous cellular reactions. DNA strand breaks, resulting either from the direct action of genotoxins on DNA or from nucleotide or base excision repair, is the cellular event common to genotoxic agents. Both *in vivo* and *in vitro*, DNA strand breaks activate poly(ADP-ribose) polymerase [PARP; NAD⁺ ADP-ribosyltransferase; NAD⁺:poly(adenosine-diphosphate-D-ribosyl)-acceptor ADP-D-ribosyltransferase, EC 2.4.2.30], an enzyme that catalyzes the synthesis of poly(ADP-ribose) from the respiratory coenzyme NAD⁺; the polymer is attached primarily to PARP itself and to a few other DNA-binding proteins (for a review, see refs. 1 and 2).

Poly(ADP-ribosylation) reactions are believed to be involved in the regulation of strand break rejoining and in cell recovery from DNA damage (3). However, the precise role of

PARP in the cellular response to genotoxic agents is not known. Most studies of the physiological role of PARP have used competitive inhibitors (benzamides and derivatives) about which there may be some reservations (4). In general, these inhibitors increase the cell sensitivity to genotoxic agents, but it has not been determined to what extent this increase results from inhibition of PARP.

PARP is a multifunctional, highly conserved enzyme that binds tightly to DNA breaks stabilizing a V-shaped DNA conformation (5). The protein (113 kDa) has a modular organization: a 46-kDa N-terminal DNA-binding domain, which acts as a molecular nick sensor, encompassing two zinc finger motifs (6) and a bipartite nuclear location signal (7); a 22-kDa central region bearing the auto-poly(ADP-ribosylation) sites, which serves to modulate PARP-DNA interaction; and a C-terminal 54-kDa catalytic domain whose activity is strongly stimulated after binding to DNA strand breaks (8).

We have shown previously that the transient expression of the DNA-binding domain (DBD) of PARP has a dominant-negative effect on the endogenous PARP activity (9, 10). Moreover, this trans-dominant inhibition of PARP, requiring the integrity of the second zinc finger, leads to a specific block of DNA repair synthesis induced by alkylation damage (base excision pathway) but not by UVC irradiation (nucleotide excision pathway) (10).

To gain further insight into the biological role of PARP in dividing cells, we have established stable cell lines constitutively producing either the wild-type (wt) PARP-DBD or a form of the DBD containing a mutation in the second zinc finger. Expression of the wt DBD had a slight effect on undamaged cells but substantially increased their sensitivity to DNA-damaging agents based on several parameters including cell growth rate, cell cycle arrest, survival, apoptosis, and sister chromatid exchanges (SCEs).

MATERIALS AND METHODS

Plasmids, Cell Culture, and Establishment of Cell Lines. Cloning of the cDNA encoding the wt or mutated PARP-DBD in the pECV23 *Xho* vector (11) has been described (10). HeLa S3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 7% fetal bovine serum and 1% gentamycin (GIBCO). Transfections were performed as described (12) with 5 µg of plasmid per 10⁶ cells. Hygromycin B (300 µg/ml; Boehringer Mannheim) selection was applied 48 h posttransfection and maintained thereafter. Resistant clones were then

pooled (HpECV) or isolated (HR138I, H46-1, and H46-4). DBD expression was assayed by Western blot analysis using a polyclonal antibody raised against the second zinc finger as described (13).

Northern and Southern Blot Analysis. Cytoplasmic RNA was prepared as described (14), transferred to nylon membrane (Hybond N; Amersham), and hybridized according to Sambrook *et al.* (15). The *Hind*III/*Hind*III fragment encoding part of the DBD (6), the *Eco*RI/*Eco*RI fragment of the glyceraldehyde-3-phosphate dehydrogenase cDNA (16), and the 1800-bp *Eco*RI/*Eco*RI fragment of hygromycin B were labeled by the random hexamer priming method (17) and used as probes. Total genomic DNA was prepared as described by Sambrook *et al.* (15). DNA (10 μ g) was digested with *Bam*HI, fractionated on 1% agarose gel, and transferred to nylon membrane (Hybond N+; Amersham).

PARP Enzyme Activity in Permeabilized Cells. Assay of PARP activity was carried out in a total volume of 100 μ l as described by Yoshihara *et al.* (18) except that NAD^+ was ^{32}P labeled (0.025 $\mu\text{Ci/nmol}$; 1 Ci = 37 GBq; NEN). PARP activity was stimulated by the addition of a double-stranded *Eco*RI linker of 8 bp to a final concentration of 20 ng/ μ l.

Cell Number Doubling Time. Exponentially growing cells (4×10^5 cells per 60-mm dish; eight dishes per cell line) were treated with various genotoxic agents and cells of two dishes were counted after 0, 1, 2, and 3 days. Doubling time was calculated from the linear portion of growth curves.

Cell Survival. Exponentially growing cells (4000 cells per 60-mm dish for H46-1, H46-4, and HR138I; 1000 cells per dish for HpECV) were treated in triplicate with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (0–8 μM ; Sigma) for 30 min at 37°C or irradiated with UVC (0–15 J/m²). After 10 days of cultivation, clones were fixed in ethanol, stained with 3% Giemsa (Aldrich), and counted.

SCEs. Cells (10^6 per 100-mm dish) were treated with 0, 2, or 6 μM MNNG for 30 min at 37°C. SCEs were then analyzed as described (19). Fifty mitoses were scored for each experiment and results are expressed as number of SCEs per chromosome.

Flow Cytometric Analysis. Flow cytometric analysis was carried out in a fluorescence-activated cell sorter (FACStar Plus; Becton Dickinson). To monitor DNA synthesis, determination of 5-bromodeoxyuridine (BrdUrd) incorporation was performed essentially as described (20), 22 h after MNNG treatment.

Apoptosis. Six hours after MNNG treatment of cells at the indicated doses, low molecular weight DNA was prepared according to Hirt (21) and treated with proteinase K (100 $\mu\text{g/ml}$) and RNase A (200 $\mu\text{g/ml}$) prior to electrophoresis on a 1% agarose gel.

RESULTS

Selection of Stable Cell Lines That Constitutively Express PARP-DBD. Several clones obtained after transfection of HeLa cells with the wt PARP-DBD construct and selection with hygromycin B were isolated and their level of DBD production was visualized by Western blot analysis (Fig. 1A) using a specific polyclonal antibody raised against the second zinc finger of the human PARP (13). Two clones (H46-1 and H46-4) showing a high level of PARP-DBD expression were expanded. Clone HR138I overproduces a mutated version of the PARP-DBD in which Arg-138 has been changed to Ile (10). This mutated peptide has no affinity for DNA *in vitro* as determined by Southwestern blot experiments (10). The HpECV control cell line was obtained by pooling the clones selected after transfection of the pECV23 *Xho* vector (11). The antibody detects the endogenous PARP and the recombinant DBD with apparent molecular masses of 116 and 46 kDa, respectively. The molar ratio of the overproduced DBD over the endogenous PARP was estimated to be 10 by Western

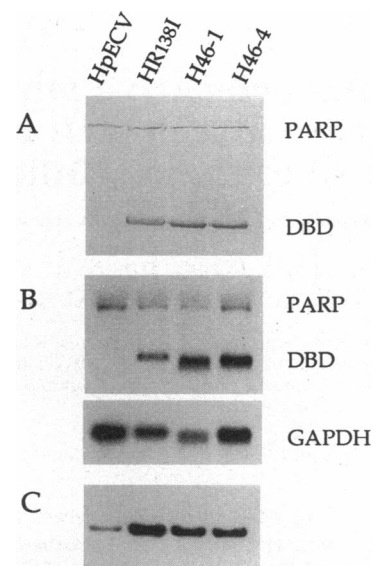


FIG. 1. Characterization of the HpECV and derivative cell lines H46-1, H46-4, and HR138I. (A) Western blot analysis using a polyclonal antibody raised against the second zinc finger of PARP (13). (B) Northern blot analysis using a PARP or a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. (C) Southern blot analysis of the episomal plasmid using a hygromycin B probe.

blotting experiments (Fig. 1A). Production of the DBD had no apparent influence on the level of endogenous PARP. By immunofluorescence analysis, we observed that >95% of the cells expressed the wt or the mutated DBD compared to the HpECV control cell line (data not shown).

Both PARP-DBD and endogenous PARP mRNAs were detected by Northern blot analysis using a fragment of PARP-DBD cDNA as a probe, thus confirming the high level of expression of PARP-DBD cDNAs compared to the endogenous PARP gene (Fig. 1B).

The Southern blot analysis displayed in Fig. 1C indicates that the average copy number of the different vectors ranged from 10 copies in HpECV to >300 in the PARP-DBD-expressing cell lines, compared to dilutions of the pECV23 *Xho* vector (data not shown). An overexposure of the autoradiogram did not show the presence of integrated copies of the plasmids (data not shown). Moreover, the plasmids were present in Hirt supernatants (21), confirming their episomal location (11).

Effect of PARP-DBD Expression on PARP Enzyme Activity. To investigate PARP inhibition in PARP-DBD-producing cells, enzymatic activity was measured in permeabilized cells in the presence of a double-stranded 8-bp oligonucleotide. The presence of oligonucleotide at 20 ng/ μ l stimulated PARP activity 30-fold in HpECV, whereas this stimulation was only \approx 10-fold in HR138I, H46-1, and H46-4, consistent with a reduced PARP activity in cells producing wt or mutated PARP-DBD. Furthermore, immunofluorescence analysis using a monoclonal antibody (10H) raised against poly(ADP-ribose) showed a very low level of polymer synthesis after DNA damage in cells expressing the PARP-DBD compared to the control (HpECV) cells (data not shown).

Cell Growth Rate and Cell Cycle Distribution. Cell growth rate, expressed as doubling time, and cell cycle distribution, as measured by flow cytometric analysis, reflect early events after DNA damage. While the doubling time of the HpECV control cells was not substantially affected, the PARP-DBD-expressing cell lines exhibited an increase in their doubling time after treatment with 3 μM MNNG (Fig. 2A). However, the cellular response to UVC appeared to be PARP independent since the four cell lines were similarly affected. These data are in agreement with those demonstrating that DNA repair

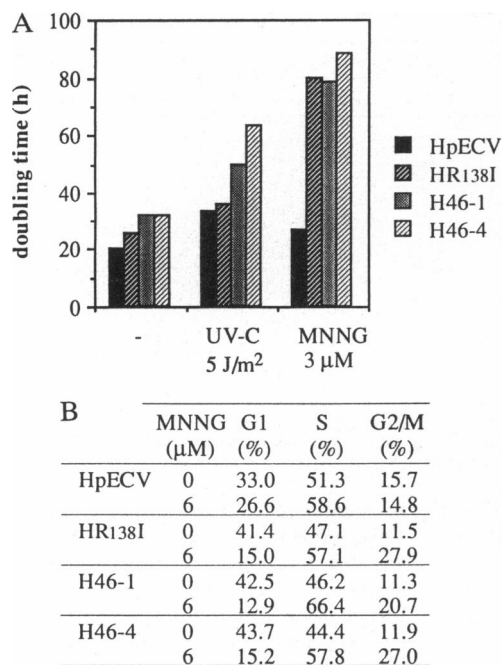


FIG. 2. Doubling time and flow cytometric analysis of the HpECV and derivative cell lines. (A) Doubling time measured after mock treatment, UVC treatment (5 J/m²), or 3 µM MNNG treatment. (B) Flow cytometric analysis of cell cycle progression in mock-treated or 6 µM MNNG-treated cell lines.

synthesis induced by alkylated damage is blocked after PARP inhibition (10, 22).

These results prompted us to examine the cell cycle distribution of each asynchronously dividing cell line 22 h after mock treatment or 6 µM MNNG exposure (Fig. 2B). As expected, the HpECV control cells treated with MNNG were able to move continuously through the cell cycle. In contrast, the three PARP-DBD-expressing cell lines exhibited an ≈ 2 -fold increase in the fraction of cells in G₂ + M after MNNG treatment. The p53-mediated G₁ arrest is absent in HeLa cells (23); however, previous studies have shown that p53-positive cell lines simultaneously exposed to 3-aminobenzamide, a potent PARP inhibitor, and to alkylating agents exhibit an accumulation in G₂ + M phase but not in G₁ (24, 25). Taken together, these results indicate that after exposure to low levels

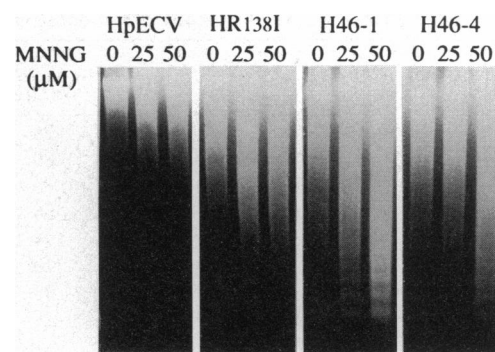


FIG. 4. Agarose gel electrophoresis of low molecular weight DNA isolated from HpECV and derivative cell lines after exposure to various doses of MNNG.

of alkylating agents, inhibition of PARP, either by 3-aminobenzamide or by production of its DBD, results in a delay in G₂ + M, independent of p53 function, strongly suggesting that PARP is critical for passing through the G₂ checkpoint.

Cell Survival and Cell Death by Apoptosis. Cell survival, as measured by colony-forming ability, was determined after MNNG or UVC treatment of cells. While MNNG treatment had a minimal effect on HpECV viability, even at a dose of 8 µM, cells expressing the wt or mutated PARP-DBD became hypersensitive (Fig. 3A). In contrast, the sensitivity of all four cell lines exposed to UVC irradiation was similar (Fig. 3B), confirming that PARP is presumably not involved in the cellular response to UVC as reported previously (10, 22).

We looked for the nucleosomal DNA ladder, one of the hallmarks of cell death by apoptosis, in order to determine whether inhibition of PARP could trigger apoptotic cell death after MNNG treatment. As shown in Fig. 4, exposure of the wt and mutated PARP-DBD-expressing cell lines to 25 or 50 µM MNNG generated the typical nucleosomal ladder of DNA fragments. The presence of apoptotic cells was confirmed by immunofluorescence microscopy using both 4',6-diamidino-2-phenylindole, to stain DNA, and the ApopTag assay, to detect DNA strand breaks (Oncor) (data not shown). In contrast, the HpECV cell line was very resistant to apoptosis after the same treatment with MNNG, as expected for a HeLa cell line. Furthermore, the cell lines expressing the PARP-DBD exhibited spontaneous apoptosis (i.e., a nucleosomal ladder was present in undamaged cells).

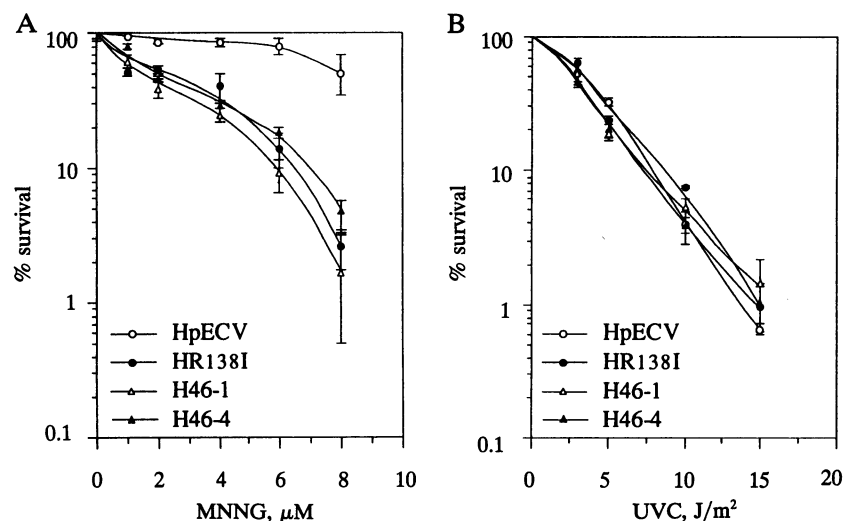


FIG. 3. Survival of HpECV and derivative cell lines after exposure to various doses of MNNG (A) and UVC (B).

Table 1. SCEs in HpeECV and derivative cell lines

	MNNG, μ M	Distribution of SCEs per chromosome							
		0–0.2	0.2–0.4	0.4–0.6	0.6–0.8	0.8–1	1–1.2	1.2–1.4	>1.4
HpeECV	0	74	26	—	—	—	—	—	—
	2	18	80	2	—	—	—	—	—
	6	—	68	32	—	—	—	—	—
HR138I	0	42	58	—	—	—	—	—	—
	2	4	74	18	2	2	—	—	—
	6	—	10	48	28	10	2	—	2
H46-1	0	18	78	4	—	—	—	—	—
	2	—	28	52	18	—	2	—	—
	6	—	—	12	14	26	22	8	18
H46-4	0	26	72	2	—	—	—	—	—
	2	—	36	44	12	4	2	—	2
	6	—	4	4	34	24	18	10	6

Results are expressed as percentage of cells presenting from 0 to >1.4 SCEs per chromosome.

Given that p53 is functionally deficient in HeLa cells, most types of lesions, including strand breaks, do not induce apoptosis. Interestingly, PARP-DBD production in HeLa cells seems to restore an apoptotic pathway, which is presumably p53 independent. It is noteworthy that HeLa cells treated with VP-16, a topoisomerase II inhibitor, undergo an apoptotic response (26). However, the signal that initiates this pathway is not known.

Effect of DBD Expression on Chromosomal Stability. SCEs are the result of recombination of replicated DNA at homologous loci. This process involves DNA strand breaks and rejoining of such breaks between sister chromatids. Cell lines expressing the wt or mutated PARP-DBD displayed a much higher frequency of SCEs than the HpeECV cell line (Table 1) both in the absence of DNA damage and after MNNG treatment. These observations are in keeping with previous results showing an increase in SCE frequency in cells treated with 3-aminobenzamide (27) and demonstrate that cells in which PARP is inhibited either by a chemical inhibitor or by its DBD present a defect in chromosomal stability that can be observed by higher spontaneous or DNA damage-induced SCE frequency.

DISCUSSION

A number of strategies have been developed to determine the physiological role of PARP in cellular responses to DNA-damaging agents. These strategies include the use of nicotinamide analogues as competitive inhibitors (reviewed in ref. 1), the generation of cell lines deficient in PARP activity obtained by random mutagenesis (18, 28, 29), and antisense RNA expression to deplete PARP in HeLa cells (30); more recently, a cell-free system has been developed to investigate the NAD-dependent repair response (22). These different approaches have pinpointed the importance of PARP in some unspecified step in the resolution of DNA strand breaks. Since dominant-negative mutants of PARP may arise naturally as a result of mutations causing premature termination of translation, in this study the N-terminal PARP-DBD was constitutively expressed in HeLa cells in order to inhibit wt PARP. The validity of this strategy was demonstrated previously in transiently transfected cells (9, 10).

PARP is an abundant protein that may also have a structural role either in the nuclear matrix (31) and/or in the maintenance of chromatin conformation. In fact, PARP depletion can also result in phenotypes related to secondary effects of the perturbed chromatin conformation (30). In the dominant-negative approach, production of the PARP-DBD does not affect the level of endogenous PARP; therefore, it seems unlikely that the phenotypes observed after MNNG exposure do not result from a side effect of chromatin conformation.

Although the molecular mechanism of the trans-inhibition of PARP by its DBD appears to occur via its DNA-binding function (9), the unexpected behavior of the HR138I cell line in the experiments described here indicates that the dominant-negative effect may also occur via another as yet unknown mechanism(s)—e.g., stabilization of the mutated peptide on the DNA strand break by the formation of a heterodimer or by a yet unknown PARP partner.

Most of our results are in agreement with previous work using PARP inhibitors (ref. 3 and references therein). However, the increased levels of apoptosis observed in the dominant-negative cell lines have not been described in studies using chemical inhibitors. In addition, incubation of the HpeECV cells with 5 mM 3-aminobenzamide did not restore the apoptotic response following DNA damage (data not shown).

Our results can be tentatively interpreted in the context of a DNA survey mechanism implicating the nick-sensor function of PARP as a part of the control of the replication fork progression when breaks are present in DNA. A number of observations indicate that PARP interacts with the replicative apparatus: (i) PARP copurifies with DNA replication forks (32), topoisomerase I (33), and DNA polymerase α (34); (ii) *in vivo* PARP modifies replication factors such as RP-A (35) and simian virus 40 T antigen (36), suggesting a potential role of PARP in the control of fork movement on damaged DNA. Perhaps PARP binding to a broken template causes a temporary arrest of semiconservative replication while DNA repair occurs. This scenario could explain why the inhibition of this process by overexpression of the PARP DBD leads to (i) an increase of the cytotoxicity of the damaging agent; (ii) an increase in SCE frequency that occurs when replication is stopped by unreparable (or poorly repairable) lesions (37); (iii) a G₂ arrest that reflects the failure to complete replication and/or repair; and (iv) induction of the apoptotic response of a cell in which one or both of these two essential functions, replication and repair, are impaired.

In conclusion, we suggest that PARP is a key element of the feedback control of the G₂ checkpoint, which prevents a cell in which the genome contains strand breaks from entering mitosis. This unique property of PARP provides a rational basis for the proposed use of specific PARP inhibitors to potentiate anticancer treatments (38). These inhibitors may be especially useful in treating p53-negative tumors, which have therefore lost the G₁ checkpoint (39).

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